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RESEARCH PAPER

Optimization of gamma radiation and sodium azide treatments for induced mutations in ginger (*Zingiber officinale* Roscoe) tissue culture

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Abstract

Ginger (*Zingiber officinale* Roscoe) is a versatile herb commonly used in cooking, food processing, medicine, and as an ingredient in various products. As an economically significant crop, ginger also has substantial export potential. This study aimed to determine the optimal dose of gamma radiation and the ideal concentration and exposure time for sodium azide treatment to induce mutations in ginger tissue culture. Ginger plantlets cultured on MS medium supplemented with 1 mg/L of BAP (6-benzylaminopurine) were subjected to acute gamma irradiation at doses of 0, 10, 20, 30, 40, and 50 Gy (dose rate: 3.37 Gy/min) using the Gamma MARK I Irradiator at the Nuclear Technology Research Center (KU-NTRC), Kasetsart University. The experiment used a completely randomized design (CRD) with three replications. The survival rate and new shoot development of the M_1V_1 generation were assessed 60 days after irradiation (DAI). At 60 DAI, a significant difference was noted, with irradiated plantlets exhibiting lower survival rates compared to the control group. The survival rate among irradiated plantlets did not differ significantly across the 20–50 Gy range, with survival percentages ranging from 55.2% to 59.4%. As a result, the $LD_{50\ (60)}$ could not be determined. However, an increased radiation dose negatively affected plantlet growth, with the new plantlet development at 60 DAI showing more significant inhibition than the control. Compared to the control, the dose causing a 50% reduction in tissue growth was identified as 16 Gy ($GR_{50(60)}$). Therefore, the optimal gamma radiation dose for inducing mutations in ginger tissue culture is between 10 and 20 Gy. For sodium azide treatment, a factorial experiment in CRD with three replications was conducted, analyzing two factors: sodium azide concentration and exposure time. The results indicated that the most effective sodium azide concentration was 0.1 mM with a treatment duration of 1–2 hours, whereas 0.3 mM was optimal for treatments shorter than 1 hour. The M_1V_2 generation exhibited phenotypic changes, including variegated and curled leaves and dwarf plant morphology, resulting from the acute gamma irradiation and sodium azide treatments, suggest the potential development of new ornamental or compact ginger varieties.

Keywords

Ginger, mutation, breeding, gamma ray, sodium azide

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Introduction

Ginger (*Zingiber officinale* Roscoe) is a widely cultivated herb with significant culinary, medicinal, and economic importance. It is prominent in global food production, health supplements, and processed products. As a high-value crop, enhancing ginger's desirable traits – such as disease resistance, yield, and quality – has become a major focus in plant breeding, particularly through genetic improvement techniques like mutation breeding (Magdy et al. 2020). Induced mutagenesis, which utilizes physical or chemical agents to induce genetic variation, has emerged as a crucial tool in developing new ginger varieties with enhanced characteristics (Abua et al. 2020). Among the mutagenic treatments, gamma radiation and sodium azide are frequently employed in plant tissue culture systems to generate beneficial mutations. Various physical and chemical mutagens are used to improve the mutation frequency through *in vivo* and *in vitro* random mutagenesis and to facilitate selections for desired traits (Mullins et al. 2021). Along with traditional selective breeding in the field, *in vitro* mutagenesis is commonly used to generate phenotypic and genotypic variations (Lu et al. 2007). The *in vitro* cell and tissue culture techniques are used alone or in combination with advanced breeding programs and offer breeders the possibility to conduct selection in a shorter time frame (Serrat et al. 2014). Tissue culture techniques of *in vitro* mutagenesis and somaclonal variation improve desired traits of a genotype without altering the other parts of the genome (Ahloowalia and Maluszynski 2001). Gamma radiation induces genetic variability by altering plant DNA, which can then be exploited for breeding improved varieties (Abdulla et al. 2009). However, the optimal dose of gamma radiation needed to effectively induce mutations in ginger while minimizing tissue damage remains an area of ongoing research. Previous studies in other crops have suggested that radiation doses between 10 and 20 Gy can effectively induce mutations without excessive tissue damage (Ahloowalia 1998). Given the potential negative effects of gamma radiation on plant growth, understanding the dose-survival relationship is essential for optimizing mutation breeding strategies for ginger. Chemical mutagens, such as sodium azide, are also commonly used to induce mutations in various plant species (Khan et al. 2010). Sodium azide functions by introducing point mutations into the plant's DNA, with its effectiveness dependent on the concentration and duration of treatment. The optimal concentration and exposure time for sodium azide vary across species and plant varieties (Gruszka et al. 2012; El-Mokadem and Mostafa 2013; El-Feky et al. 2014). Nevertheless, determining the ideal concentration and treatment duration specific to ginger tissue culture remains challenging. This study aims to investigate the effects of gamma radiation and sodium azide treatment on ginger tissue cultures. Specifically, it seeks to determine the optimal gamma radiation dose, the ideal sodium azide concentration, and the treatment duration for inducing mutations. By exploring these factors, this research aims to determine the optimal dose of gamma radiation and the ideal concentration and exposure time of sodium azide treatment that effectively induce mutations in ginger tissue culture and support the development of improved ginger varieties through mutation breeding.

Materials and methods

Plant materials

Pathogen-free plantlets of ginger (*Zingiber officinale* Roscoe) were cultured in MS medium (Murashige and Skoog 1962) supplemented with 1 mg/l BAP (6-benzylaminopurine), 3% sucrose, and 2.5 g/l gelatin until more than 1000 plantlets were produced for the experiments.

Acute gamma irradiation

A completely randomized design (CRD) experiment was conducted, with six treatment groups based on varying radiation doses. Each treatment included three replications, with 20 plants per replication. Initially grown on MS media, eight-week-old ginger tissue cultures were subjected to acute gamma irradiation at 0 (control), 10, 20, 30, 40, and 50 Gy. The irradiation was administered using a Gamma Mark I irradiator (gamma chamber) at the Nuclear Technology Research Center, Faculty of Science, Kasetsart University, with a dose rate of 3.37 Gy/min. Following irradiation, the tissues were transferred to fresh MS media, with this generation of tissue culture designated as M_1V_1. Data on the number of surviving plants, dead plants, and growth performance (assessed by counting the number of new shoots) were recorded 60 days post-irradiation. The survival and growth percentages were calculated, and these data were used to determine the radiation dose causing 50% mortality ($LD_{50(60)}$) and the dose causing a 50% reduction in growth ($GR_{50(60)}$) compared to the control group (non-irradiated).

Sodium azide treatment

Eight-week-old ginger plantlets in the fresh media were treated with sodium azide to induce mutation. The experiment followed a factorial design in a Completely Randomized Design (CRD) with two factors: Factor 1: Sodium azide concentration, which included four levels: 0 mM (control), 0.1 mM, 0.3 mM, and 0.5 mM; and Factor 2: Duration of sodium azide exposure, which consisted of three times: 1 hour, 2 hours, and 3 hours. After exposure to the sodium azide treatment, the plant tissues were transferred to MS media for recovery. This generation of tissue culture was designated as M_1V_1. At 60 days post-treatment, data were collected on the number of surviving plants, dead plants, and growth performance, which was assessed based on the number of new shoots. The survival and

growth percentages were calculated, and the data were analyzed to determine the optimal sodium azide concentration and exposure time for inducing mutations.

Mutation selection

After recording and calculating the initial data for the M_1V_1 generation, the surviving new plantlets were subcultured onto fresh MS medium for the M_1V_2 generation. These plantlets were then monitored for any morphological changes compared to the control. New plantlets from the M_1V_2 generation were subsequently transferred to new media for further growth. Desirable variations were carefully documented and selected for further analysis.

Experimental design and statistical analysis

The collected data were analyzed using Analysis of Variance (ANOVA) based on a Completely Randomized Design (CRD) and a Factorial in CRD approach. The differences in means were compared using the Least Significant Difference (LSD) method. All statistical analyses were performed using the R software (Jompuk 2012).

Results and discussion

Effect of acute gamma irradiation on survival and growth percentage in tissue culture of Ginger in the M_1V_1 generation

At 60 days after gamma irradiation, survival and growth rate data were collected for the M_1V_1 generation. Following this, new plantlets were transferred to fresh MS medium bottles, called the M_1V_2 generation. Morphological changes in the new plantlets were observed, and putative mutants were separated from normal-looking plantlets. The survival percentage of ginger tissue culture showed a highly significant difference ($p < 0.01$). Tissues irradiated at all doses exhibited lower survival percentages compared to the unirradiated control. Specifically, the survival percentages for doses between 20 and 50 Gy were not statistically different, with the lowest observed survival percentage at 55.2% as a percent of control.. Thus, although irradiation clearly had an effect on survival rate, the exact $LD_{50(60)}$ value could not be calculated (Table 1, Figs 1, 2). This finding aligns with those of Sharma et al. (2020), who investigated gamma radiation's effect on ginger tissue culture to select for *Fusarium oxysporum* f. sp. zingiberi resistance. Their study showed plant mortality at 56, 19, and 15 Gy at 4, 12, and 16 weeks, respectively. Another study by Sharma and Thakur (2021) reported survival rates of 95% after exposure to 10 Gy down to 12.33% following exposure to 100 Gy. Similarly, Tosri et al. (2019) found that higher doses of gamma radiation (Cs-137) reduced the survival rate of *Curcuma alismatifolia* tissues. Rashid et al. (2013) observed that increasing gamma radiation doses resulted in reduced survival rates of ginger rhizomes, while Priya et al. (2014) and Taheri et al. (2014) reported similar findings in turmeric and various Pumila rhizomes, respectively.

Table 1. Survival and growth rate percentage of *Zingiber officinale* Roscoe *in vitro* plantlets at 60 days after gamma irradiation compared with the control.

Dose (Gy)	Survival percentage at 60 days after irradiation (as % of control)	Growth rate percentage at 60 days after irradiation (as % of control)
0	100 [a 1/]	100 [a 1/]
10	95.3 [b]	89.3 [b]
20	59.4 [c]	20.0 [c]
30	55.8 [c]	1.5 [d]
40	55.2 [c]	0 [d]
50	56.4 [c]	0 [d]
F-test	**	**
$LSD_{0.01}$	4.31	3.93
C.V. (%)	3.5	13.7

** Significant at 1% level.
[1/] Different letters in the same column mean that the data are statistically different from the least significant difference ($p < 0.01$).

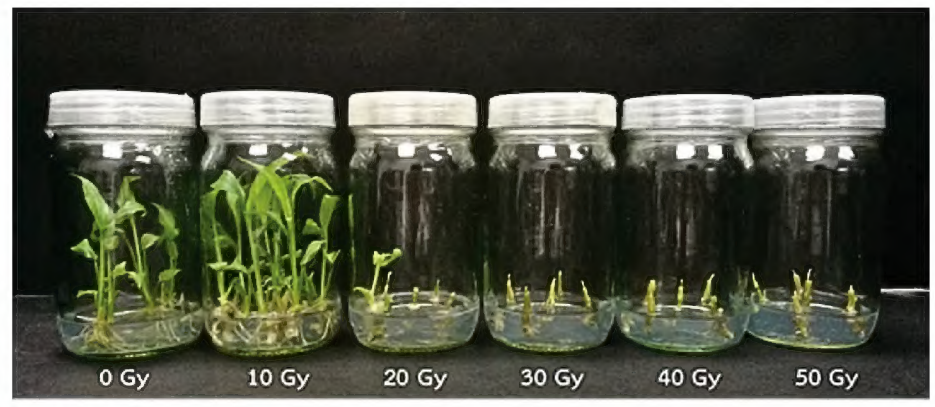

Figure 1. Control and irradiated samples at 60 d after irradiation: Control (0 Gy); gamma irradiation, 10, 20, 30, 40, and 50 Gy.

Regarding growth, acute gamma radiation significantly impacted the growth of ginger tissue culture at 60 days post-irradiation. As the radiation dose increased, the growth percentage measured by the number of new shoots decreased. Statistical analysis revealed a significant difference ($p < 0.01$) in growth percentage among the various radiation doses (Table 1). Specifically, the lowest radiation dose of 10 Gy , the growth percentage was only 89.3%, compared to the control, while at 20 Gy, the average number of new shoots decreased to just 20.0% compared to control. At the higher doses of 30, 40 and 50 Gy, the growth percentages for these treatment groups did not significantly differ from each other, but all the irradiated ginger plants exhibited extremely stunted growth, with zero or close to zero new shoots compared to control. You can see the growth rate of ginger tissue clearly decreased with increasing radiation doses. The $GR_{50(60)}$ value was found to be 16 Gy (Fig. 2). These results are consistent with Abdullah et al. (2018), who observed a decrease in the growth rate of ginger (*Zingiber officinale*) at higher gamma radiation doses in the Bentong variety. However, the Tenjung Sepat variety showed increased growth at a 5 Gy dose. Similarly,

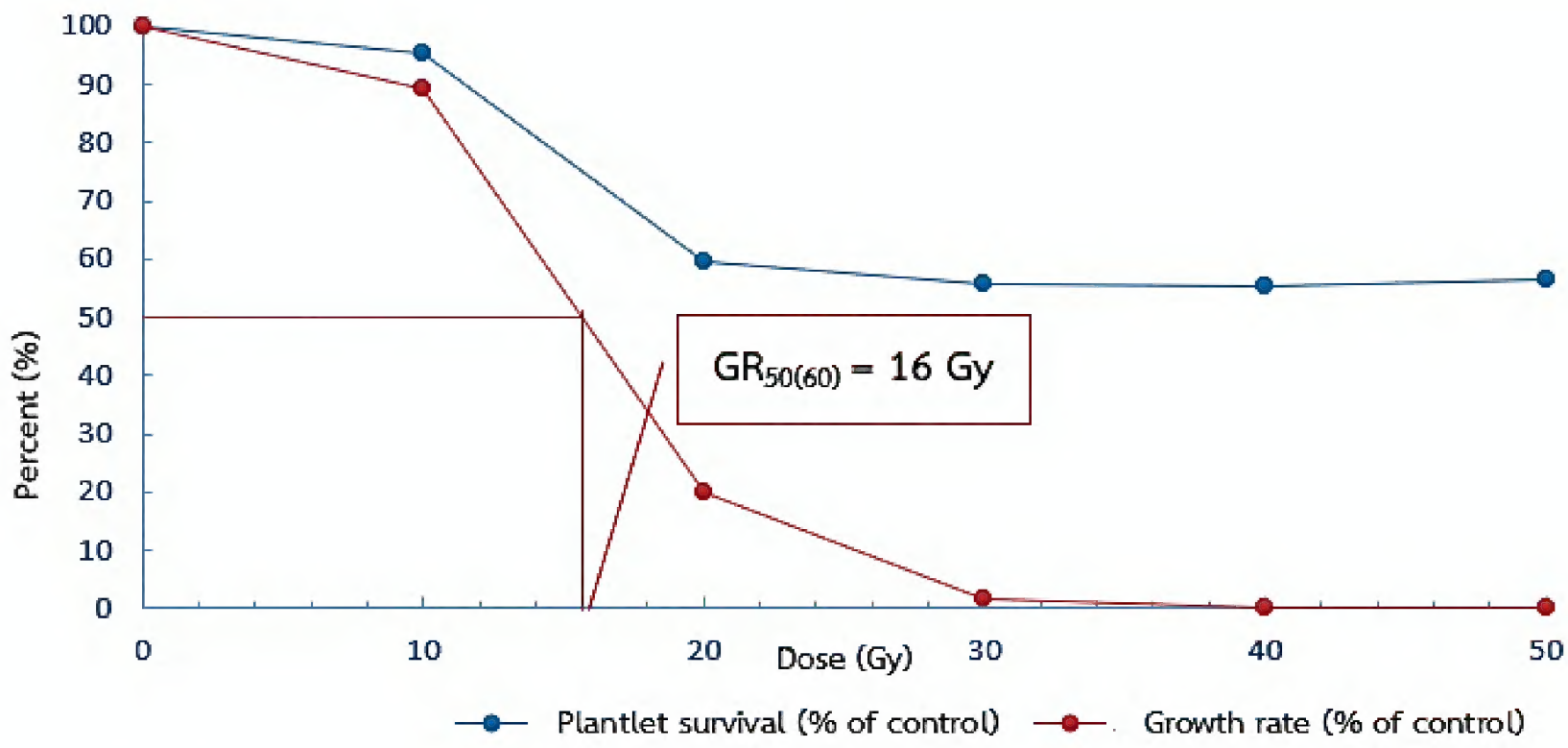

Figure 2. Effects of radiation dose on survival percentage and growth rate percentage of ginger in the M_1V_1 generation at 60 days after acute gamma irradiation.

Taheri et al. (2016) reported that higher doses of chronic gamma radiation inhibited the growth of *Curcuma alismatifolia*, affecting the number of roots, leaves, and flowers in all studied varieties. Jompuk et al. (2009) studied the effects of acute gamma radiation on *Cryptocoryne wendtii* 'Brown,' and Limtiyayotin et al. (2018) worked on *Exacam affine* Balf. f. ex Regel *in vitro* and found that the growth rate decreased with increasing radiation dose. High radiation doses are believed to inhibit growth by damaging meristematic tissues or disrupting cell division (Zhu et al. 2008; Joshi-Saha et al. 2015; Yadav 2016).

Effect of sodium azide mutagenic chemical on survival and growth rate in tissue culture of ginger in the M_1V_1 generation

Eight-week-old ginger plantlets were treated with sodium azide to induce mutations after being transferred to fresh media. The experiment utilized a factorial design in a completely randomized design (CRD), divided into two main factors: sodium azide concentration, which included four levels (0 mM, 0.1 mM, 0.3 mM, and 0.5 mM), and exposure time, comprising three periods (1, 3, and 5 hours). After treatment, the plant tissues were transferred to MS media. The experimental results were recorded at 60 days post-treatment. The results indicated that both higher sodium azide concentrations and extended exposure times led to a decrease in survival percentage. The highest survival was observed at a 1-hour exposure time, with no statistically significant differences noted between 2- and 3-hour exposures (Table 2). Based on the data, optimal parameters for mutation induction were identified: for 0.1 mM concentration, exposure should be limited to 1–2 hours; for 0.3 mM or higher, exposure time should not exceed 1 hour, as no surviving plants were found at longer durations. This result aligns with earlier studies by Türkoğlu et al. (2022) and Wannajindaporn (2015), who reported that sodium azide concentrations between 0.1 and 4 mM effectively induce mutations without severe damage in tissue cultures of wheat and *Canscora decurrens* Dalz.. When evaluating growth percentage, a similar trend was observed: as sodium azide concentration and exposure time increased, the growth of ginger tissue cultures decreased. The highest growth was achieved at a 1-hour exposure, with no significant differences between 2- and 3-hour treatments (Table 2). For 0.1 mM concentration, exposure should remain within 1–2 hours; for 0.3 mM, no new shoots were recorded at longer durations (Fig. 3). At 0.5 mM, despite some plant survival, no new growth was observed. These findings corroborate results from Gómez et al. (2019), who noted that increasing sodium azide concentrations negatively impacted growth metrics in pineapple tissue cultures.

Optimal gamma radiation dose for mutation induction

When analyzing the survival and growth percentages data, it was determined that the survival percentage , or lethal dose, as has been conventionally used, was not suitable for identifying the optimal radiation dose for mutation induction in this species, as all doses tested resulted in survival rates exceeding 50%. However, the growth percentage could be utilized to establish the appropriate radiation dose. Based on the data collected 60 days post-irradiation, the $GR_{50(60)}$ value representing the radiation dose that induces a 50% reduction in growth was calculated to be 16 Gy. In the M_1V_2 generation, morphological changes such as variegated and curled leaves were observed at 10 and 20 Gy radiation doses. Thus, the optimal gamma radiation dose for mutation induction is

Table 2. Survival and growth rate percentage of *Zingiber officinale* Roscoe *in vitro* plantlets treated with sodium azide in M_1V_1 generation at 60 days after chemical treatment.

Concentration (mM)	Survival percentage at 60 days after NaN_3 treatment (as % of control)				Growth rate percentage at 60 days after NaN_3 treatment (as % of control)			
	time (h)				time (h)			
	1	2	3	mean	1	2	3	mean
0	100 a	100 a	100 a	100 a [1/]	100 a	100 a	100 a	100 a [1/]
0.1	61.11 b	27.78 c	0 d	29.63 b	85.28 a	44.72 b	0 c	43.33 b
0.3	29.17 c	0 d	0 d	9.72 c	50.14 b	0 c	0 c	16.71 c
0.5	33.33 c	0 d	0 d	11.11 c	0 c	0 c	0 c	0 d
mean	55.90 A	31.94 B	25 B		58.85 A	36.18 B	25 B	
F-test								
concentration (C)		**				**		
time (T)		**				**		
C × T		*				**		
C.V.(%)		15.27				18.5		

* Significant at 5% level
** Significant at 1% level
[1/] Different letters in the same column mean that the data are statistically different from the least significant difference ($p < 0.05$).

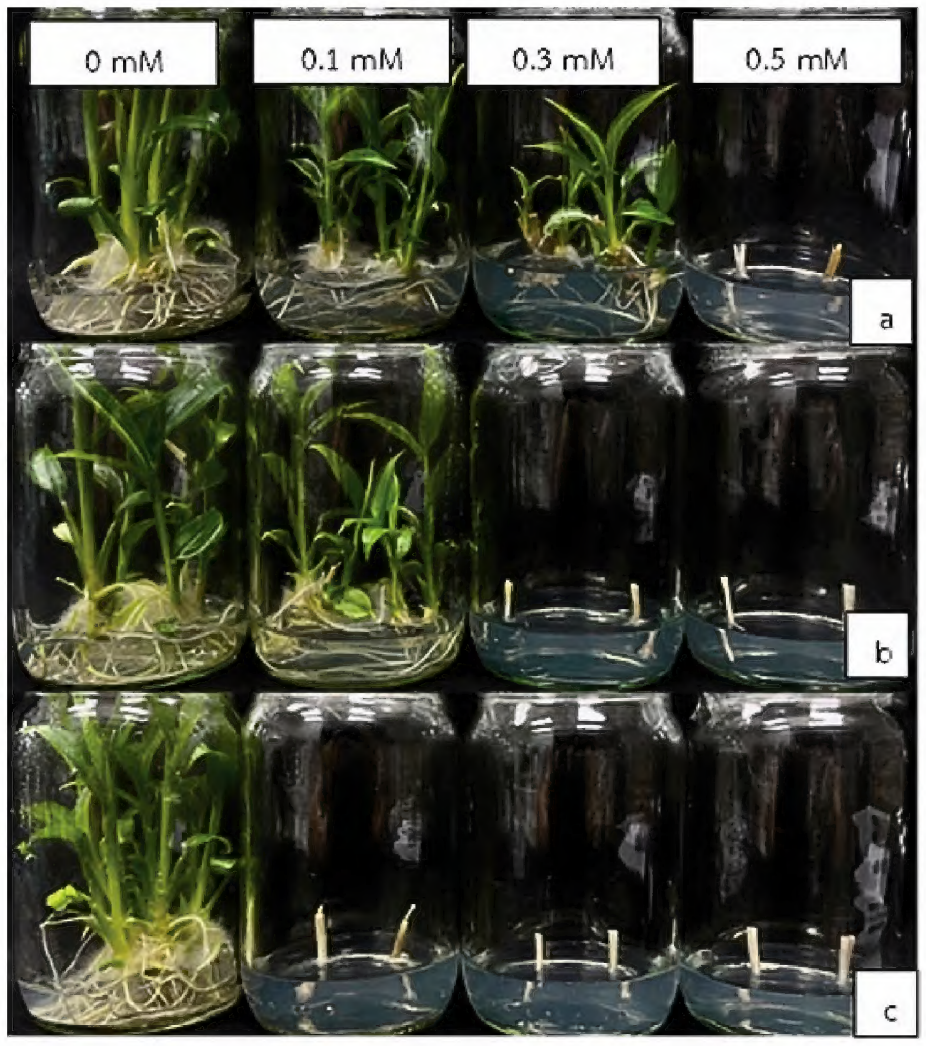

Figure 3. Ginger tissue culture at 60 days after treatment with sodium azide at different concentrations and with different treatment durations (a. 1 h, b. 2 h, and c. 3 h) compared to the control.

suggested to be between 10 and 20 Gy. This conclusion aligns with the findings of Lamseejan et al. (1996), who investigated mutation induction in red ginger (*Alpinia purpurata*) tissue cultures. In their study, they irradiated the tissue cultures of red ginger with gamma rays at doses of 0, 10, 30, 50, 70, and 90 Gy before culturing them on MS media supplemented with 2 mg/l BAP (6-benzylaminopurine) to promote shoot formation. They determined that the optimal gamma radiation dose for mutation induction was 20 Gy. At a dose of 10 Gy, they noted characteristics such as numerous white streaks on green leaves, dwarfism, and wrinkled green leaves.

Optimal sodium azide Concentration and exposure time for mutation induction

In the mutagenic chemical sodium azide experiment, the survival and growth of M_1V_1 generation ginger tissue cultures were assessed at concentrations of 0, 0.1, 0.3, and 0.5 mM, with exposure times of 1, 2, and 3 hours. The findings indicated the optimal sodium azide concentration and exposure time for inducing mutations, based on survival and growth percentages: At a concentration of 0.1 mM, the exposure time should be between 1 and 2 hours. However, when the concentration is increased to 0.3 mM, the exposure time should not exceed 1 hour. Additionally, it was noted that the growth percentage decreased as both the sodium azide concentration and exposure time increased. These results align with the studies conducted by El-Feky et al. (2014) and Gómez et al. (2019).

Morphological changes observed in the M_1V_2 generation after acute gamma irradiation

Several morphological changes were observed in the M_1V_2 generation following the acute gamma irradiation of ginger tissue cultures. At a radiation dose of 10 Gy, these included curled leaves, dwarfism, and variegated leaves. These traits, if proven to be genetically stable in subsequent generations, hold promise for the development of novel ornamental ginger cultivars or compact varieties suited for high-density cultivation. Additionally, at a radiation dose of 20 Gy, curled leaves and light green leaves

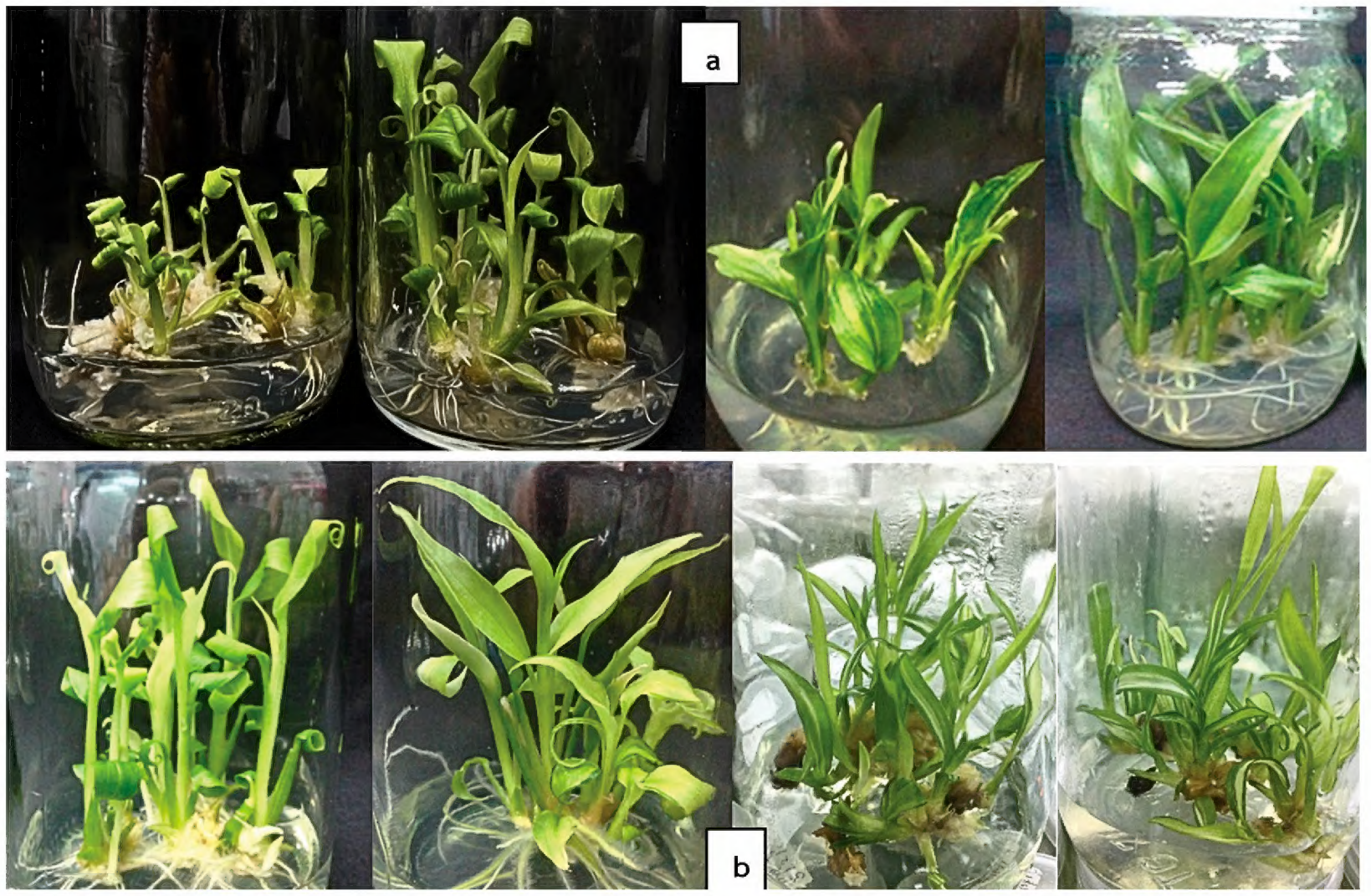

Figure 4. Morphological variation in the M_1V_2 generation of *Zingiber officinale* Roscoe: gamma irradiation. **a.** Curled leaves, dwarfism and variegated leaves at a dose of 10 Gy; **b.** Curled leaves, light green leaves and variegated leaves at a dose of 20 Gy.

were also noted (Fig. 4). These results align with the studies conducted by Raju et al. (1980), Lamseejan et al. (1996) and Tosri et al. (2019).

Morphological changes observed in the M_1V_2 generation after sodium azide treatment

In the M_1V_2 generation following sodium azide treatment of ginger in the laboratory, abnormal characteristics such as curled leaves and dwarfism were observed at concentrations of 0.1 and 0.3 mM at some exposure times (Fig. 5).

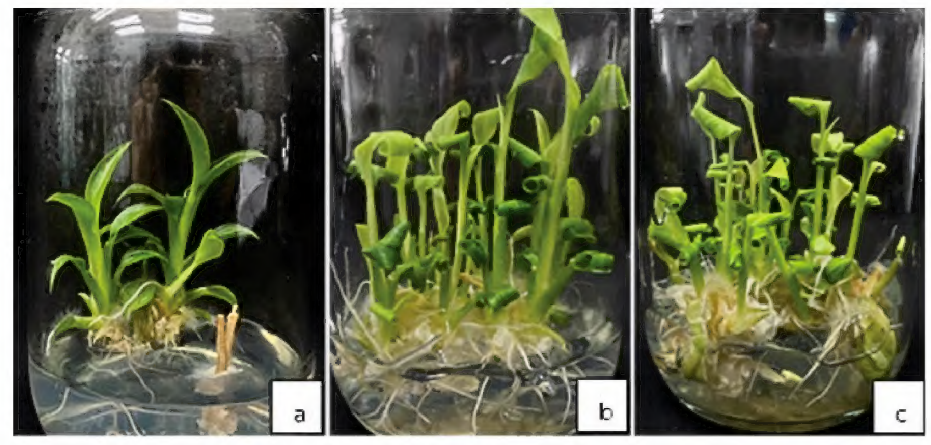

Figure 5. Morphological variation in the M_1V_2 generation of *Zingiber officinale* after treatment with sodium azide chemical at a concentration of 0.1 mM for treatment periods of 1 h (a), 2 h (b), and 0.3 mM for treatment period 1 h (c).

Conclusions

The optimal gamma radiation dose for inducing mutations in ginger tissue cultures, at a dose rate of 3.37 Gy/min, is recommended to be between 10 and 20 Gy. For sodium azide treatment, the ideal concentration and exposure time for ginger tissue cultures should range from 0.1 to 0.3 mM based on survival and growth percentages. Specifically, when using a concentration of 0.1 mM, the exposure time should be between 1 and 2 hours. If a concentration of 0.3 mM is used, the exposure time should not exceed 1 hour. In the M1V2 generation, morphological abnormalities such as curled leaves, dwarfism, and variegated leaves were observed following both treatments. These stable morphological traits suggest potential for the development of new ornamental ginger cultivars, pending confirmation through further generational evaluation.

Author contributions

Peeranuch Jompuk designed the study, conducted the experimental work, and analyzed the data. PJ wrote and revised the manuscript. Natnichaphu Sukin, Laksamon Soithong and Chayanid Saisanguan are the scientists who take care of the instruments and tissue culture lab. Choosak Jompuk analyzed the data and revised the manuscript.

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